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Visualization of Differential Gene Expression by Improved Cyan Fluorescent Protein and Yellow Fluorescent Protein Production in *Bacillus subtilis*

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The distinguishable cyan and yellow fluorescent proteins (CFP and YFP) enable the simultaneous in vivo visualization of different promoter activities. Here, we report new cloning vectors for the construction of *cfp* and *yfp* fusions in *Bacillus subtilis*. By extending the N-terminal portions of previously described CFP and YFP variants, 20- to 70-fold-improved fluorescent-protein production was achieved. Probably, the addition of sequences encoding the first eight amino acids of the N-terminal part of ComGA of *B. subtilis* overcomes the slow translation initiation that is provoked by the eukaryotic codon bias present in the original *cfp* and *yfp* genes. Using these new vectors, we demonstrate that, within an isogenic population of sporulating *B. subtilis* cells, expression of the *abrB* and *spoIIA* genes is distinct in individual cells.

The use of the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has proven to be a powerful method to study in vivo gene expression in a broad range of hosts (2). Mutagenesis of *gfp* resulted in variants with different fluorescent properties (4, 14). The use of the distinguishable cyan (*cfp*) and yellow (*yfp*) variants of *gfp* has allowed studies of multiple cellular processes within a single cell (6, 8). One of the best-studied microbial organisms displaying cellular differentiation is the gram-positive bacterium *Bacillus subtilis*. Because of its ability to develop natural competence, secrete large quantities of proteins, and form highly resistant spores, it has been extensively studied and used as a model for bacterial cellular differentiation (5, 7, 19). Therefore, the availability of easily detectable variants of CFP and YFP in *B. subtilis* would considerably facilitate studies of multiple expression patterns in the organism.

Previously reported vectors for the production of fluorescent-protein fusions in *B. subtilis* contain the genes *ecfp* (Clontech) and *eyfp* (Clontech) (8) (in this work, we will refer to these genes as *cfp* and *yfp*, respectively). However, these fusions frequently display no or weak fluorescent signals when expressed in *B. subtilis* (reference 20 and this work). Here, we show that the *cfp* and *yfp* variants described are not efficiently translated in *B. subtilis* when used in promoter-*cfp* or -*yfp* fusions. In contrast to *gfp* (18), the codon usage in the *cfp* and *yfp* genes has been optimized for use in eukaryotic cell lines (8). Although a strong bias in codon usage has not been observed for *B. subtilis* (22), it was reported that, particularly at the initial stages of translation, the occurrence of less preferred triplets has an effect on translation efficiency (11, 27, 30, 32). Moreover, highly expressed genes of *B. subtilis* generally display a codon usage significantly different from that of genes expressed at low levels (22).

In order to obtain stable and efficiently translated variants of CFP and YFP in *B. subtilis*, vectors encoding CFP and YFP variants having an N-terminal extension were constructed. This N-terminal extension contains the first eight amino acids of ComGA, a strongly expressed *B. subtilis* protein involved in competence development (12). Our present studies show that the addition of this N-terminal extension overcomes the hampering of the initiation and processivity of translation. As a result, high levels of fluorescent protein can be produced.

Studying the underlying mechanisms of the differentiation of an isogenic population into distinct developmental stages is an important task in developmental biology. The new vectors described in this paper allow the visualization of differential gene expression within a genetically identical population. In this respect, the process of sporulation in *B. subtilis* has been studied for many years as a model for cellular differentiation. A major role of Spo0A, the key sporulation regulator, is to repress the expression of *abrB* and activate the transcription of the *spoIIA* operon (for a review, see reference 31). By visualizing the expression of the *abrB* and *spoIIA* promoters using the CFP- and YFP-encoding vectors described in this work, we demonstrate that within an isogenic population of *B. subtilis* cells, the initiation of sporulation is distinct from expression of the *abrB* promoter, which is observed in nonsporulating cells. These results demonstrate the practicability of the novel vectors for studying bacterial cellular differentiation.

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MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. TY (tryptone-yeast extract) medium contained Bacto-Tryptone (1%), Bacto-Yeast Extract (0.5%), and NaCl (1%). Sporulation medium contained dehydrated nutrient broth (0.8%), NaOH (0.5 mM), $MgSO_4$ (1 mM), KCl (1 g/liter), $Ca(NO_3)_2$ (1 mM),

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant properties	Source or reference
E. coli MC1061	F$^-$ *araD*139 Δ(*ara-leu*)7696 Δ(*lac*)X74 *galU galK hsdR2 mcrA mcrB1 rspL*	Laboratory stock
B. subtilis		
168	*trpC2*	16
86-IIA	168; P_{spoIIA}-*cfp* Cm^r	This study
87-IIA	168; P_{spoIIA}-*yfp* Cm^r	This study
icfp-IIA	168; P_{spoIIA}-*icfp* Cm^r	This study
iyfp-IIA	168; P_{spoIIA}-*iyfp* Cm^r	This study
icfp-IIA-amyE	168; *amyE*::P_{spoIIA}-*icfp* Km^r	This study
86-abrB	168; P_{abrB}-*cfp* Cm^r	This study
87-abrB	168; P_{abrB}-*yfp* Cm^r	This study
icfp-abrB	168; P_{abrB}-*icfp* Cm^r	This study
iyfp-abrB	168; P_{abrB}-*iyfp* Cm^r	This study
iyfp-abrB-icfp-IIA-amyE	168; P_{abrB}-*iyfp* Cm^r *amyE*::P_{spoIIA}-*icfp* Km^r	This study
Plasmids		
pSG1186	*bla cat cfp*	8
pSG1187	*bla cat yfp*	8
pDK	*bla amyE' bgaB kan 'amyE*	36
pICFP	*bla cat icfp*	This study
pIYFP	*bla cat iyfp*	This study
p86-IIA	*bla cat* P_{spoIIA}-*cfp*	This study
p87-IIA	*bla cat* P_{spoIIA}-*yfp*	This study
pICFP-IIA	*bla cat* P_{spoIIA}-*icfp*	This study
pIYFP-IIA	*bla cat* P_{spoIIA}-*iyfp*	This study
pAmy-ICFP-IIA	*bla amyE'* P_{spoIIA}-*icfp kan 'amyE*	This study
p86-abrB	*bla cat* P_{abrB}-*cfp*	This study
p87-abrB	*bla cat* P_{abrB}-*yfp*	This study
pICFP-abrB	*bla cat* P_{abrB}-*icfp*	This study
pIYFP-abrB	*bla cat* P_{abrB}-*iyfp*	This study

$MnCl_2$ (0.01 mM), and $FeSO_4$ (0.001 mM). S7 medium was prepared as described by van Dijl et al. (34). Minimal medium for *B. subtilis* was prepared as described by Leskela et al. (17). When required, medium for *Escherichia coli* was supplemented with ampicillin (100 μg/ml); media for *B. subtilis* were supplemented with chloramphenicol (CHL; 5 μg/ml) or kanamycin (KAN; 10 μg/ml).

Recombinant DNA techniques and oligonucleotides. Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of *E. coli* were carried out as described by Sambrook et al. (28). Enzymes were obtained from Roche (Mannheim, Germany). *B. subtilis* was transformed as described by Leskela et al. (17). The oligonucleotides used in this study are listed in Table 2.

Plasmids. To construct the plasmid pICFP carrying the "improved" *cfp* gene (*icfp*), a PCR with the primers cfp-yfp-comG-F+EcoRI and RnlacZ-fw (Table 2) was performed, using the plasmid pSG1186 (*cfp*) (8) as a template. To construct the plasmid pIYFP carrying the improved *yfp* gene (*iyfp*), the same primer pair was used in a PCR using pSG1187 (*yfp*) (8) as a template. Plasmid pSG1186 contains the *cfp* gene derived from pECFP (Clontech), and pSG1187 contains the *yfp* gene derived from pEYFP-C1 (Clontech). The amplified fragments were subsequently cleaved with EcoRI and XbaI and ligated into the corresponding sites of pSG1186, replacing the *cfp* gene with the *icfp* and *iyfp* genes. This resulted in plasmids pICFP and pIYFP, respectively. Sequencing of the multiple cloning sites of pICFP and pIYFP showed that the *cfp* gene corresponded to the original gene, whereas the *yfp* gene contained a single (A → G) point mutation in nucleotide 557, resulting in an Asp186 → Gly186 (D186G) substitution. However, this point mutation does not appear to affect the fluorescence spectrum or intensity of IYFP compared to those of the previously reported YFP.

To construct plasmids p86-IIA and p87-IIA, carrying the *B. subtilis spoIIA* promoter region fused with the *cfp* or *yfp* gene, a PCR with the primers IIA-F and IIA-R (Table 2) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with KpnI and ClaI and ligated into the corresponding sites of pSG1186 and pSG1187, resulting in plasmids p86-IIA and p87-IIA, respectively.

To construct plasmids pICFP-IIA and pIYFP-IIA, carrying the *B. subtilis spoIIA* promoter region fused with the *icfp* or *iyfp* sequence, a PCR with the primers IIA-F-500+KpnI and pSpoIIAA-R-HindIII (Table 2) was performed using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with KpnI and HindIII and ligated into the corresponding sites of pSG1186 and pSG1187, resulting in plasmids pICFP-IIA and pIYFP-IIA, respectively. It should be noted that the first 24 bp of *comGA* were included in the pSpoIIAA-R-HindIII primer.

To construct plasmid pAmy-ICFP-IIA, plasmid pICFP-IIA was cleaved with KpnI and XbaI. The resulting 1.3-kb fragment, carrying the P_{spoIIA}-icfp fusion, was ligated into the corresponding sites of pDK (36), resulting in the plasmid pAmy-ICFP-IIA. Note that as a result of this cloning strategy, the P_{spoIIA}-icfp region replaced the *bgaB* gene present on pDK.

To construct plasmids p86-abrB, p87-abrB, pICFP-abrB, and pIYFP-abrB, a PCR with the primers F-abrB and R-abrB (Table 2) was performed, using chromosomal DNA of *B. subtilis* 168 as a template. The amplified fragment was subsequently cleaved with ClaI and EcoRI and ligated into the corresponding sites of pSG1186, pSG1187, pICFP, and pIYFP to generate plasmids p86-abrB, p87-abrB, pICFP-abrB, and pIYFP-abrB, respectively.

Strains. *B. subtilis* strains 86-IIA, 87-IIA, icfp-IIA, and iyfp-IIA were obtained by a Campbell-type integration (single crossover) of plasmids p86-IIA, p87-IIA, pICFP-IIA, and pIYFP-IIA into the chromosomal *spoIIA* promoter region of *B. subtilis* 168. Transformants were selected on TY agar plates containing CHL after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

B. subtilis strain cfp-IIA-amyE was obtained by a double-crossover recombination event between the *amyE* regions located on pAmyE-ICFP-IIA and the chromosomal *amyE* gene of *B. subtilis* 168. Transformants were selected on TY agar plates containing KAN after overnight incubation at 37°C. Correct integration into the *amyE* gene was tested and confirmed by a lack of amylase activity upon growth on plates containing 1% starch.

B. subtilis strains 86-abrB, 87-abrB, icfp-abrB, and iyfp-abrB were obtained by a Campbell-type integration of plasmids p86-abrB, p87-abrB, pICFP-abrB, and pIYFP-abrB into the chromosomal *abrB* promoter region of *B. subtilis* 168. Transformants were selected on TY agar plates containing CHL after overnight incubation at 37°C. Correct integration was verified by PCR (data not shown).

TABLE 2. Oligonucleotides

Oligonucleotide	Sequence (5′ to 3′)[a]	Description; position
cfp-yfp-comG-F+EcoRI	CGGAATTCTTGGATTCAATAGAAAAGGTAAGCGAATTTGC CACCATGGTGAGCAAGGGCGAGGAG	EcoRI; 24 bp of *comGA*; 5′ end of *cfp*/*yfp*
RnlacZ-fw	GGTTTTCCCAGTCACGACGTTGTAA	3′ end of *lacZ′*
IIA-F	GGGGTACCAGGCCAAGAGCTTGGCACT	KpnI; 5′ end of P_{spoIIA}
IIA-R	CCATCGATGAGGCTCATGCTCATTCCTCCTTG	ClaI; 3′ end of P_{spoIIA}
IIA-F-500+KpnI	GGGGTACCCGAACTCACTACATCTGAGCCG	*KpnI*; 5′ end of P_{spoIIA}
pSpoIIAA-R-HindIII	CCCAAGCTTGCTTACCTTTTCTATTGAATCCAAGCTCATTC CTCCTTGATATGATCG	HindIII; 24 bp of *comGA*; 3′ end of P_{spoIIA}
F-abrB	CCATCGATCGGCATCTTGAAACCTCCTA	*Cla*I; 5′ end of P_{abrB}
R-abrB	GGAATTCCTTCATAAACATTCTCCTCCC	EcoRI; 3′ end of P_{abrB}
gfp1	TGCTCTAGAGGCATGGTGAGCAAGGGCGAGGA	XbaI; 5′ end of *gfp*/*cfp*/*yfp*

[a] Relevant restriction sites are underlined.

B. subtilis strain iyfp-abrB-icfp-IIA-amyE was obtained by transformation of strain iyfp-abrB with chromosomal DNA of strain icfp-IIA-amyE. Transformants were selected on TY agar plates containing CHL and KAN after overnight incubation at 37°C.

Microscopy. Cells were prepared for microscopy and applied to agarose slides as described by Glaser et al. (9), and images were acquired using an Axiophot microscope equipped with an AxioVision camera (Zeiss, Oberkochen, Germany). Fluorescence filter sets used to visualize CFP and YFP were obtained from Zeiss. Fluorescent signals of CFP were visualized using set 47 (excitation, 426 to 446 nm; emission, 460 to 500 nm), and fluorescent signals of YFP were visualized using set 46 (excitation, 490 to 510 nm; emission, 520 to 550 nm). AxioVs20 software (Zeiss) was used for image capture, and the figures were prepared for publication using Corel Graphics Suite 11. The ICFP protein displays a fluorescence excitation maximum of 434 nm and an emission maximum of 477 nm. The IYFP protein displays a fluorescence excitation maximum of 514 nm and an emission maximum of 527 nm. CFP fluorescence cannot be visualized using the YFP filter, and likewise, fluorescence of YFP cannot be visualized using the CFP filter (data not shown).

Western blot analysis and immunodetection. Cells were separated from the growth medium by centrifugation (20,800 × *g*; 1 min; room temperature). The pelleted cells were resuspended in protoplast buffer (20 mM potassium phosphate, pH 7.5, 15 mM $MgCl_2$, 20% sucrose, and 1 mg of lysozyme/ml) and incubated at 37°C for 30 min. The resulting protoplasts were diluted with 2× sodium dodecyl sulfate (SDS) sample buffer, incubated at 95°C for 5 min, and separated by SDS-polyacrylamide gel electrophoresis (PAGE) as described previously (28). Next, the proteins were transferred to a polyvinylidene difluoride membrane (Roche) as described previously (28). CFP and YFP were detected with polyclonal anti-GFP antibodies (Molecular Probes, Leiden, The Netherlands) and horseradish peroxidase-anti-rabbit immunoglobulin G conjugate (Amersham Biosciences, Little Chalfont, United Kingdom) according to the manufacturers' instructions. Anti-GFP antibodies can be used to detect CFP and YFP due to high amino acid sequence conservation among GFP, CFP, and YFP (15).

Fluorimetric analysis of total cytosolic protein extracts. Cells were separated from the growth medium by centrifugation (10,600 × *g*; 2 min; room temperature). The pelleted cells were washed with and resuspended in 50 mM Tris-HCl, pH 7. Next, 0.5 g of glass beads (50- to 105-μm diameter) were added, and the cells were disrupted using a minibeadbeater (twice for 1 min each time; BioSpec Products, Bartlesville, Wash.). To remove the glass beads, samples were centrifuged (20,800 × *g*; 5 min; 4°C), and the supernatants were transferred to clean 0.5-ml tubes. Cytosolic proteins were separated from membranes by velocity centrifugation (195,000 × *g*; rotor TLA-120.1; 30 min; 4°C). Samples were analyzed on a fluorimeter (LS-50 B; Perkin-Elmer, Boston, Mass.) using quartz cuvettes (101 QS; Hellma, Müllheim, Germany). The settings to measure CFP fluorescence were as follows: excitation, 436/10; emission, 480/20. The settings to measure YFP fluorescence were as follows: excitation, 500/10; emission, 535/15. During all measurements, the photomultiplier tube voltage was set at 750 V.

Protein labeling, immunoprecipitation, SDS-PAGE, and fluorography. Pulse-chase labeling of *B. subtilis*, immunoprecipitation, SDS-PAGE, and fluorography were performed as described previously (34). Immunoprecipitations were performed with specific antibodies against GFP (Molecular Probes).

RNA isolation and RNA dot blotting. Exponentially growing cells were collected by centrifugation, and RNA was extracted as described previously (35). The RNA quantity was spectrophotometrically measured on an ND-1000 (Nanodrop Technologies, Wilmington, Del.), and the RNA quality was checked by capillary electrophoresis using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, Calif.). For the production of a radioactively labeled probe, a DNA fragment containing the *yfp* gene was amplified by PCR using primers gfp1 and RnlacZ-fw (Table 2) and using the plasmid pIYFP as a template. The resulting 847-bp fragment was radiolabeled using the BioPrime random-labeling kit (Invitrogen) essentially as described by the manufacturer, with the exception of using [α-^{32}P]dCTP instead of biotin-labeled CTP. The radiolabeled DNA fragments were purified using a Sephadex G-25 spin column (Amersham). RNA dot blotting was performed as follows. Volumes (1.0 μl) of a dilution series of purified RNA were spotted onto a positively charged nylon membrane (Boehringer Mannheim, Mannheim, Germany), which was subsequently wrapped in aluminum foil and baked for 45 min at 120°C. Hybridization of the radiolabeled DNA probe to the spotted membrane was performed using the DIG Northern Starter kit (Roche) hybridization buffer. The membrane was prehybridized in 20 ml of hybridization buffer for 2 h at 50°C in a roller bottle. Subsequently, the radiolabeled probe was added to 4 ml of fresh hybridization buffer and denatured by boiling it for 5 min. The prehybridization buffer was removed from the bottle, and the denatured probe was added and hybridized to the membrane for 18 h at 50°C. After being washed (two times with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]–0.1% SDS for 5 min each time at room temperature and two times with 0.1× SSC–0.1% SDS for 15 min each time at 50°C), the membrane was air dried and exposed overnight to a phosphorimager screen (Packard BioScience, Meriden, Conn.). Readouts were performed using a Cyclone machine (Packard).

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited at GenBank (pICFP, AY653731; pIYFP, AY653732).

RESULTS AND DISCUSSION

Construction of new *cfp* and *yfp* variants. Promoter fusions with the unmodified *cfp* or *yfp* gene in *B. subtilis* resulted in little or no production of fluorescent protein and thus poor in vivo fluorescence (see below). For this reason, we set out to construct improved variants of *cfp* and *yfp*. To obtain vectors carrying improved variants of *cfp* and *yfp*, the first 24 bp of the coding sequence of *comGA* were fused to the *cfp* and *yfp* genes. The N terminus of ComGA was selected, because this protein is produced at high levels during competence development (1) and was shown to be highly stable when used in fusions with CFP or YFP (data not shown). Using a primer carrying sequences encoding the first eight amino acid residues of ComGA (Table 2), the *cfp* and *yfp* genes were amplified and cloned into pSG1186 (8), replacing the original *cfp* gene with the extended *cfp* or *yfp* gene (see Materials and Methods). Using this cloning strategy, the original EcoRI recognition site of pSG1186 was removed and a new EcoRI recognition site was introduced upstream of the *comGA* sequence. Due to introduction of the *comGA* sequence, a TTG start codon was employed instead of the ATG start codon present in the original *cfp* or *yfp* sequence. The use of the non-ATG start codon TTG is quite common in gram-positive organisms (1) and does not seem to have a substantial influence on translation efficiency (24). A schematic presentation of the pICFP and pIYFP vectors is given in Fig. 1. The pICFP and pIYFP plasmids contain a multiple cloning site directly upstream of the TTG start codon. This allows the construction of C-terminal fluorescent protein fusions and/or promoter activity studies. The *E. coli* strains containing either pICFP or pIYFP (pICFP, ECE180; pIYFP, ECE181) can be ordered from the *Bacillus* Genetic Stock Center (http://www.bgsc.org/).

CFP and YFP are inefficiently produced in *B. subtilis*. To compare fluorescent-protein production from fusions made with the previously described vectors (8) and the vectors described above (Fig. 1), the *B. subtilis abrB* and *spoIIA* promoter regions were fused with the fluorescent-protein-encoding genes (see Materials and Methods). Expression driven from these promoters was shown to be high under specific growth conditions (29). The promoter fusion plasmids were introduced in *B. subtilis* 168 and integrated into the chromosome of the organism. Next, strains were examined for fluorescent-protein production by fluorescence microscopy and Western blotting. *B. subtilis* strains 86-abrB (P_{abrB}-*cfp*), icfp-abrB (P_{abrB}-*icfp*), 87-abrB (P_{abrB}-*yfp*), and iyfp-abrB (P_{abrB}-*iyfp*) were grown in TY medium, and samples were withdrawn at the mid-exponential growth phase. In addition, strains 86-spoIIA (P_{spoIIA}-*cfp*), icfp-IIA (P_{spoIIA}-*icfp*), 87-spoIIA (P_{spoIIA}-*yfp*), and iyfp-IIA (P_{spoIIA}-*iyfp*) were grown in sporulation medium, and cells were harvested 2 h after entry into the stationary growth phase. The cells were analyzed by fluorescence micros-

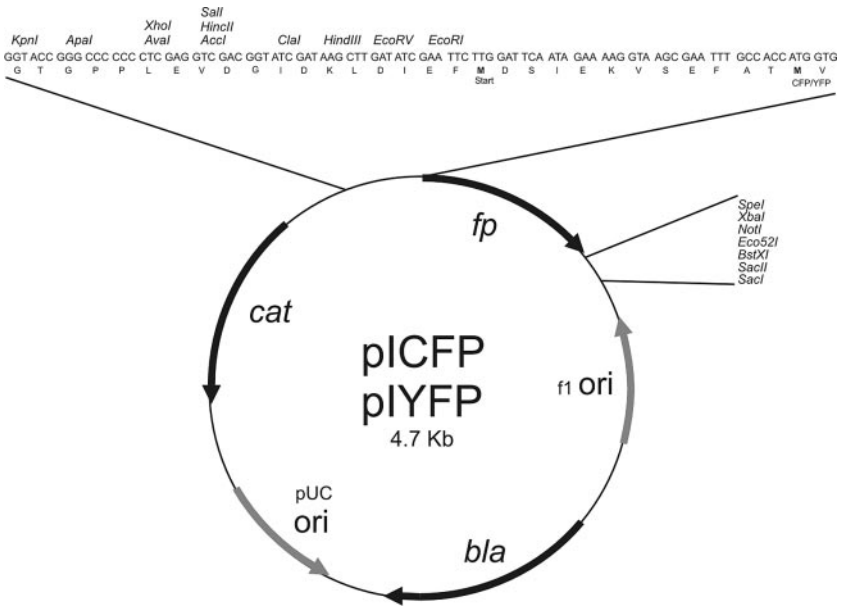

FIG. 1. Schematic presentation of plasmids pICFP and pIYFP. Unique restriction sites are depicted. The reading frame upstream of the fluorescent-protein-encoding gene is shown. *bla*, β-lactamase; *cat*, chloramphenicol acetyltransferase; ori, origin of replication; *fp*, gene encoding a fluorescent protein (pICFP harbors the *icfp* gene; pIYFP contains the *iyfp* gene).

copy using the appropriate filters. Strikingly, only strains harboring the *comGA-cfp* and *comGA-yfp* fusions showed detectable fluorescence signals (Fig. 2). Therefore, we refer to these variants as improved *cfp* and *yfp* (*icfp* and *iyfp*). As shown by Western blotting, only minor amounts of CFP and YFP could be visualized in cells containing the original variants, whereas strains expressing the N-terminally extended variants (ICFP and IYFP) showed considerably higher protein production levels (Fig. 3).

To quantify the differences in fluorescent signals shown in Fig. 2, the total fluorescence in cytosolic protein extracts was determined using a fluorimeter. The different *B. subtilis* strains containing the *abrB*-promoter fusions were grown in TY medium, and samples were taken at the mid-exponential growth phase. The *abrB* fusion strains were chosen for this experiment, since *abrB* is highly expressed during exponential growth. Total cytosolic proteins were isolated, and fluorescence was measured as described in Materials and Methods. As specified in Table 3, ICFP protein extracts showed 20- to 30-times-higher fluorescence than CFP extracts. For IYFP, an improvement of between 50 and 70 times could be measured compared to the fluorescence of YFP.

Taken together, these results demonstrate that production of the modified fluorescent proteins in *B. subtilis* is considerably higher and is sufficient to be visualized by fluorescence microscopy, in contrast to the unmodified variants.

Production of *cfp* and *yfp* mRNAs in *B. subtilis*. To investigate whether the small amounts of CFP and YFP proteins produced in *B. subtilis* resulted from low mRNA production levels, RNA dot blot experiments were performed. The different *B. subtilis* strains containing *abrB*-promoter fusions were grown in TY medium, and samples were withdrawn at the mid-exponential growth phase. RNA was isolated, blotted, hybridized, and analyzed as described in Materials and Methods. As shown in Fig. 4, production levels of *cfp* and *yfp* mRNAs did not differ significantly from the levels produced by the *icfp* and *iyfp* variants. This result suggests that the insufficient production of CFP and YFP in *B. subtilis* is not related to an inadequate production of *cfp* and *yfp* mRNAs.

Increased translational efficiencies of ICFP and IYFP in *B. subtilis*. To investigate the stability and putative degradation of CFP/YFP and ICFP/IYFP, pulse-chase labeling experiments were performed. The different *B. subtilis* strains containing the *abrB*-promoter fusions were grown in S7 medium, and cells were labeled with [^{35}S]methionine-[^{35}S]cysteine for 30 s prior to a chase with an excess of nonradioactive methionine-cysteine. As depicted in Fig. 5, the fluorescent proteins with an N-terminal extension (ICFP and IYFP) are produced more rapidly and in larger quantities than CFP and YFP. While both the ICFP and IYFP proteins were already produced at high levels immediately after the chase of the cells, even small amounts of CFP and YFP were not detectable at that point. As already demonstrated by Western blotting (Fig. 3), significant degradation of the fluorescent proteins could not be observed. This indicates that proteolytic activity by one of the endogenous proteases of *B. subtilis* is probably not the cause of the low levels of CFP and YFP compared to the ICFP and IYFP protein levels. More likely, the low levels of CFP and YFP production were the result of the low translation efficiencies of the original *cfp* and *yfp* genes.

***abrB* and *spoIIA* are distinctly expressed.** To demonstrate the experimental applicability of the vectors described in this work, we examined the expression of the *abrB* and *spoIIA* genes within an isogenic population of *B. subtilis* cells. In *B. subtilis*, transition state regulator proteins play an essential role in the adaptive capacity and survival of the cell. The transcription regulator AbrB regulates many stationary-phase processes by repressing the expression of genes involved in sporulation

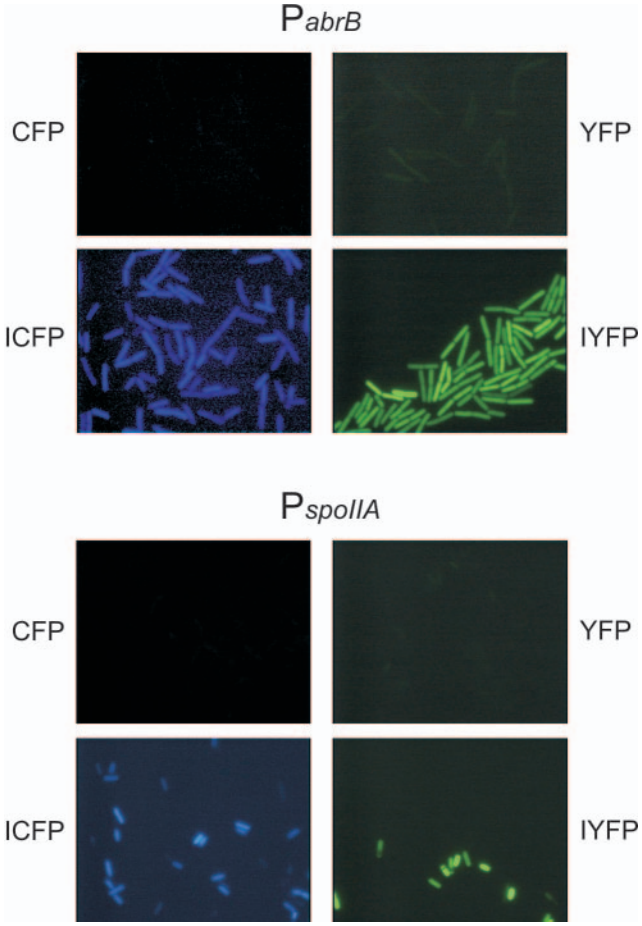

FIG. 2. Visualization of fluorescent-protein production in *B. subtilis* by fluorescence microscopy. Strains carrying an *abrB* promoter-*cfp* or -*yfp* fusion were grown in TY medium, and samples were withdrawn at mid-exponential growth phase (upper panels). Strains containing a *spoIIA* promoter-*cfp* or -*yfp* fusion were grown in sporulation medium, and cells were collected 2 h after entry into the stationary growth phase (lower panels). Production of CFP, YFP, ICFP, or IYFP, whose expression was driven by activity of either the *abrB* (P_{abrB}) or the *spoIIA* (P_{spoIIA}) promoter, was visualized by fluorescence microscopy, as described in Materials and Methods.

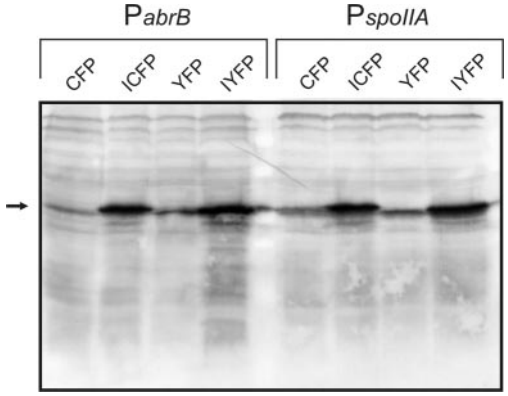

FIG. 3. Fluorescent-protein production. Strains carrying an *abrB* and *spoIIA* promoter-*cfp* or -*yfp* fusion were grown as described in the legend to Fig. 2. Cells were separated from the growth medium by centrifugation and analyzed by SDS-PAGE and Western blotting using GFP-specific polyclonal antibodies. The arrow indicates GFP-specific signal.

(e.g., *spo0E* and *spoVG*), competence (e.g., *comK*), degradative enzyme production (e.g., *aprE*), amino acid utilization (e.g., *dpp*), and antibiotic production (e.g., *tycA*) (13, 26, 33). When cells reach the end of exponential growth and various environmental signals promote the activation of the response regulator Spo0A, *abrB* expression is repressed by Spo0A~P (23, 25). Furthermore, Spo0A~P activates >40 genes directly, including the *spoIIA* operon, which contains the early sporulation genes *spoIIAA*, *spoIIAB*, and *sigF* (21). Chung et al. proposed that expression of the *spoIIA* operon (and initiation of sporulation in general) requires a threshold concentration of Spo0A~P (3). Knowing this, it is to be expected that cells that initiate sporulation (i.e., express *spoIIA*) do not express *abrB*. To see whether expression of *abrB* and initiation of sporulation are strictly separated between individual cells, we investigated how the expression of *abrB* and *spoIIA* is distributed between cells within an isogenic population. Since cells that have initiated sporulation (and have not yet formed an asymmetric septum) are hardly distinguishable from other cells by light microscopy, a *B. subtilis* strain was constructed that enabled visualization of the activity of the *abrB* and *spoIIA* promoters at a single-cell level. We constructed a strain in which the *iyfp* gene is under the control of the *abrB* promoter (integrated at the *abrB* promoter region) and the *icfp* gene is under the control of the *spoIIA* promoter (integrated at the *amyE* locus). The resulting double-labeled strain was grown overnight in TY medium, and cells were collected for analysis by fluorescence microscopy. As shown in Fig. 6, only part of the population produces IYFP expressed from the *abrB* promoter. In these cells, production of ICFP expressed from the *spoIIA* promoter cannot be observed. Accordingly, cells expressing ICFP from the *spoIIA* promoter do not express IYFP from the *abrB* promoter. It should be noted that in some cells that predominantly express ICFP, a weak signal in the yellow filter could be detected. This signal most likely represented residual IYFP protein that was produced due to *abrB* promoter activity at earlier growth stages and that is maintained in the cell as a result of its stable nature.

During exponential growth, all cells were shown to express IYFP from the *abrB* promoter (data not shown). Since AbrB is a repressor of many stationary-phase processes, this is not surprising. At the beginning of the stationary growth phase, expression of ICFP from the *spoIIA* promoter can be observed (data not shown). Interestingly, this expression was not ob-

TABLE 3. Fluorescence determinations of cytosolic protein extracts

Sample	Fluorescence[a] at:		
	1:100	1:50	1:10
CFP	ND	0.9	8
ICFP	ND	26.4	165
YFP	0.9	ND	11
IYFP	69.2	ND	568

[a] Fluorescence is presented in arbitrary units. The results of three dilutions of cytosolic protein extracts are shown. Samples were normalized against a cytosolic protein extract of the parental *B. subtilis* 168 strain and were prepared and measured as described in Materials and Methods. ND, not determined.

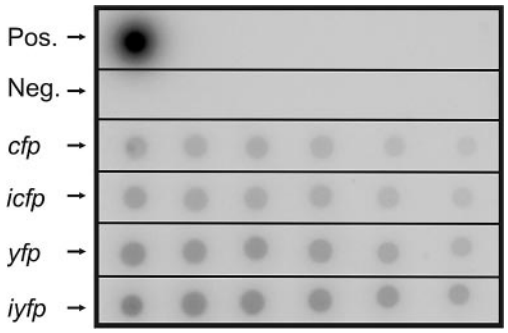

FIG. 4. Production of CFP-, ICFP-, YFP-, and IYFP-encoding mRNAs. Strains 168, 86-abrB (P_{abrB}-*cfp*), icfp-abrB (P_{abrB}-*icfp*), 87-abrB (P_{abrB}-*yfp*), and iyfp-abrB (P_{abrB}-*iyfp*) were grown in TY medium, and samples for RNA isolation were withdrawn at the mid-exponential growth phase. Twofold serial dilutions of total RNA, starting with 4.5 μg, were applied to the membrane and probed as described in Materials and Methods. As a positive control (Pos.), 30 ng of unlabeled PCR fragment was spotted. Total RNA from the parental *B. subtilis* 168 strain was spotted as a negative control (Neg.).

served in all cells, indicating that initiation of sporulation is a heterogeneous process. This was also demonstrated in a different way by flow cytometry experiments (3). Gonzalez-Pastor and coworkers (10) showed that cells that have initiated sporulation development can delay the commitment to further stages of sporulation by killing their siblings and utilizing the nutrients that are released. The two operons involved in this so-called self-digestion (*skf* and *sdp*) are directly regulated by active Spo0A, as is the *spoIIA* operon (21, 29). The cannibalistic behavior of a sporulating culture could, at least in part, account for the observed heterogeneity in *spoIIA* expression. We are currently trying to understand the underlying mechanisms involved in this heterogeneous process.

Concluding remarks. We have constructed new *cfp* and *yfp* vectors encoding fluorescent proteins with an eight-amino-acid N-terminal extension that can be produced at useful levels in *B. subtilis*, and probably also in other high-AT gram-positive bacteria. Our results indicate that the presence of the sequence encoding this N-terminal extension overcomes the impairment

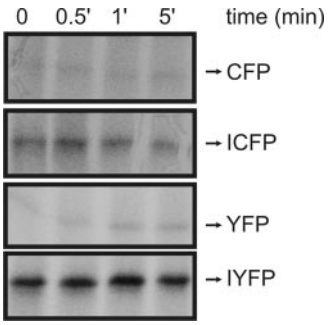

FIG. 5. Production and stability of fluorescent proteins in *B. subtilis*. Strains 86-abrB (P_{abrB}-*cfp*), icfp-abrB (P_{abrB}-*icfp*), 87-abrB (P_{abrB}-*yfp*), and iyfp-abrB (P_{abrB}-*iyfp*) were grown in S7 medium, and cells were labeled with [^{35}S]methionine-[^{35}S]cysteine for 30 s prior to a chase with an excess of nonradioactive methionine-cysteine. Samples were withdrawn at the indicated times and trichloroacetic acid precipitated. Proteins were immunoprecipitated using anti-GFP antibodies, and samples were used for SDS-PAGE and fluorography.

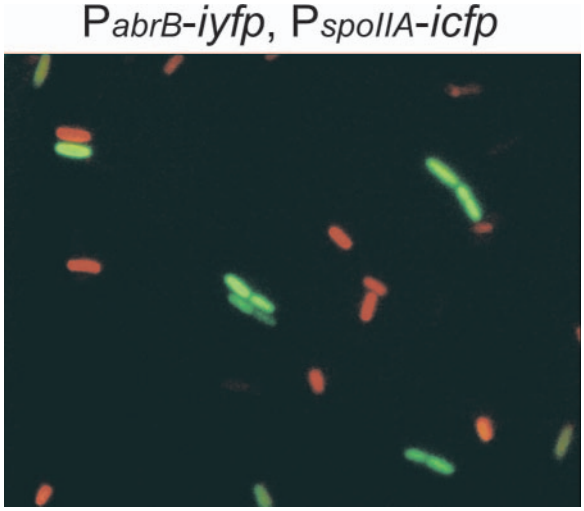

FIG. 6. Production of ICFP and IYFP, driven by the *abrB* and *spoIIA* promoters in an isogenic *B. subtilis* culture. Strain iyfp-abrB-icfp-IIA-amyE (P_{abrB}-*iyfp*, *amyE*::P_{spoIIA}-*icfp*) was grown overnight in TY medium, and cells were collected for analysis by fluorescence microscopy. IYFP and ICFP images were combined in the green and red channels, respectively. Green cells produce IYFP, whose expression is driven by activity of the *abrB* promoter; red cells represent production of ICFP, whose expression is driven by activity of the *spoIIA* promoter.

of translation that is provoked by the human codon bias present in the original *cfp* and *yfp* genes. This shows that the codon usage in the initial sequence of a gene can play an important role in the production of that protein in *B. subtilis*. By extending a (heterologous) gene with a sequence encoding a stable and highly expressed protein of *B. subtilis*, the production of this (heterologous) protein can be significantly increased when expressed in *B. subtilis* and probably also in other high-AT gram-positive bacteria.

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